Award Number: W81XWH-13-1-0270

TITLE: Validation of Biomarkers of the Tumor Microenvironment

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REPORT DATE: October 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                        Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                          Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual	30 Sep 2013 - 29 Sep 2014

4. TITLE AND SUBTITLE

"Validation of Biomarkers of the Tumor Microenvironment"

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0270

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Irvine
Medical Sciences D440
Irvine, Ca 92697-4800

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goals of the first year of the funding period were to develop methods for the analysis of FFPE (formalin-fixed paraffin-embedded) prostate cancer biopsy tissue in order validate the accuracy of a stroma-based classifier for diagnosis of prostate cancer using FFPE anatomically negative biopsies. The methods that needed to be developed were PCR primers that operate on RNA retrieved from FFPE prostate cancer tissue and to apply the primers as a microarray to accurately detect RNA corresponding to the primer targets. Proof-of-principal for these goals has been achieved.

15. SUBJECT TERMS
Prostate cancer/formalin-fixed paraffin-embedded/diagnosis/microenvironment/stroma/validation

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION:

The goal of the first year of the funding period (9/30/13-9/29/14) was to develop methods for the analysis of FFPE (formalin-fixed paraffin-embedded) prostate cancer biopsy tissue in order to validate the accuracy of a multigene stroma-based classifier for diagnosis of prostate cancer using anatomically negative FFPE biopsies during year 2. The methods that needed to be developed were the design of PCR primers that operate on RNA retrieved from FFPE prostate cancer tissue and to apply the primers as a microarray to accurately detect RNA corresponding to the primer targets in FFPE tissue. These goals have been completed. Two microarrays, also termed "cards" here, were created using primer sequences that met the proposed standards (**Table 1**). The RNA isolated for analysis from FFPE prostate cases was characterized and found to be of sufficient quality for PCR. The prognosis array was validated on prostate cancer cases with known outcome as either post-surgery recurrent cases or post-surgery nonrecurrent cases. 30 genes of the genes of the array were selected by objective criteria (PAM program (Predicative Analysis for Microarrays; 10-fold cross validation) which were used to form a 30 gene FFPE Prognosis Classifier with an accuracy of 96%. Thus, we are able to form FFPE classifiers from for FFPE prostate cancer tissue and provides a proof-of-principle for the original goal.

2. KEYWORDS: Prostate cancer/formalin-fixed paraffin-embedded/tissue/diagnosis /microenvironment/stroma/validation/multigene classifier/

3. OVERALL PROJECT SUMMARY

Background. Conversion of biomarkers to qPCR assays on FFPE biopsies samples. We have developed a diagnostic (1) and prognostic (2) assays based on analysis of Affymetrix gene expression arrays which were hybridized with RNA from *fresh frozen* prostate cancer tissue. Both projects utilized tumor-adjacent stroma or microenvironment tissue. The Diagnostic Classifier utilized tissue of known diagnosis while the Prognostic Classifier utilized tissue from prostate cancer cases with known clinical outcome of either having undergone post-surgery recurrence or were known to be recurrence free for at least five years post-surgery. Genes selected as members of the final classifiers are based on the use of a 10-fold cross validation selection process as implemented with the program Prediction Analysis for Microarrays or PAM. The overall goal is to convert the PAM classification method to utilize qPCR values from patient biopsy tissue, *i.e.* from FFPE tissue. **Aim 1**, year 1, is the retraining step. Conversion utilizes testing which of the genes

Table 1: Numbers of genes represented on PCR CARDS and final numbers of genes selected by 10-fold cross validation (PAM) as a classifier for prostate cancer using fresh or FFPE tissue.

FUNCTION	TRAINING CARD[1,3.] FFPE ONLY (primer sets/card)	PAM-Selected CLASSIFIER[2.]	
		FFPE	FROZEN TISSUE (probe sets)
Diagnosis	89 (4)	N/A	114 (131)
Prognosis	186 (2)	30	15 (19)

1."CARD" denotes a 384 well microfluidics card preprinted with primer pairs and TaqMan reagents;
2. PAM (Prediction Analysis for Microarrays), utilizes 10-fold cross validation for selection of genes from a starting set such as all the genes on the training card to derive the gene set of a classifier.
3. The training cards include additional primer pairs for 3-6 housekeeping genes with each set of experimental primer pairs.

used for frozen tissue classifier works well on FFPE RNA. In the case of the Prognostic Classifier, many alternative genes also were included in order to allow for the possibility that not all genes of the original frozen tissue classifier are suitable for measuring RNA from FFPE tissue.. These genes are included in the fabrication of 384-well plate microfluidics cards for PCR of FFPE RNA. The numbers of genes in the

original classifiers and on the training cards for migration to FFPE classifiers is summarized in **Table 1. Progress is summarized below in alignment with the Statement of Work.**

Statement of Work (SOW) (quoted *verbatim*).

Specific Aim 1: Aim 1 is to migrate a current fresh frozen tumor assay to use with formalin-fixed paraffin-embedded tissue for clinical use.

Major Task 1: Task 1. Migrate the current Diagnostic Classifier for use with Formalin-fixed Paraffin-embedded (FFPE) tissue.

Subtask 1: Developing PCR Primer Probes for FFPE Samples. In silico development of PCR primers for use on FFPE (tissue).

Progress for Subtask 1. This task is completed. Primer development started with primer design for FFPE tissue. Prior studies revealed the Affymetrix probe sets that were used to develop a 114 multigene frozen tissue diagnostic classifier (1). These probe sets were developed with frozen tissue (1). The design of PCR primers for assay of the same genes in FFPE tissue was largely a bioinformatics step starting with using the Affymetrix Inc. web-based information to determine sequences of the successful probe sets. These sequences were used in primer design with the three following design criteria: (1) include sequences of the probe sets of the Affymetrix array used in the current 114 probe set, (2) select those probe sets that either span or extend the sequence choice to span splice junctions of the target gene and (3) select primer pairs for these sequences with amplicons of about 75 bp and less than 125 bp. Sequences that fit these criteria were screened in the 1.2 million primer pairs of the data base of ABI Life Technologies as recommended to work with FFPE RNA.

The subtask was extended. We developed two sets of primer pairs (**Table 1**). In the case of the 114-gene diagnostic classifier, 89 pairs were selected based on the 89 best performing genes of the diagnostic classifier using PAM weights. Second we developed FFPE primer pairs for our published 15-gene prognostic classifier. The classifier predicts recurrence of disease following surgery with intent-to-cure. This classifier has advantages for validating the migration of a classifier from frozen tissue to FFPE tissue (below). 186 primer pairs were chosen. The large excess over the original 15 genes derived from the frozen tissue analysis was to provide many alternatives for replacing members of the 15-gene set should the corresponding primer pairs not function well with FFPE-derived RNA. . The alternative gene identities were taken from the gene list submitted to PAM in deriving the 15-gene classifier. As reviewed below, 30 genes of the 186 genes were required to create an FFPE prognostic classifier, *i.e.* >> 15 genes.

The selected pairs have been applied to Life Technology microfluidics cards together with PCR reagents for TaqMan amplification. Each 384 well card contains two sets of primer pairs for analysis of two RNA preparations.

Subtask 2: Selection of Preserved RNA Targets.

Tissue used: Formalin-fixed Paraffin Embedded tissue blocks of prostate cancer retrieved from the UCI Pathology archives. The cases to be used correspond to cases collected by the NCI SPECS consortium centered at UCI (PI-D. Mercola) by informed consent 2005-2009 (the UCI NCI SPECS resources).

Progress for Subtask 3. The subtask was completed. The required FFPE blocks of prostate cancer cases was retrieved and utilized as described below.

Subtask 3: Evaluation of Degradation by comparison of amount of selected RNA targets in FFPE and fresh frozen tissue as the gold standard both from the same cases. Tissue used: (as for Subtask 2).

Progress for Subtask 3. This subtask is essentially complete. This subtask was undertaken in modified form. A 30 gene prognostic classifier was developed based on the results of applying our 186 primer pair microfluidics card to 25 FFPE RNA samples (retrieved as per **Subtask 2**) from prostate cancer of known outcome. The classifier was then tested on 47 independent prostate cancer cases with known outcome where the arrays were hybridized with RNA isolated from *frozen tissue* as explained in greater detail below (see **Bioinformatics Testing, Task 2, Subtask 2**). Briefly, the FFPE classifier performed on independent frozen tissue RNA with a classification accuracy = 0.82, sensitivity = 0.64, specificity = 0.95. These excellent results indicate that FFPE RNA supports the selection of genes that form classifiers that accurately work on RNA from frozen tissue.

Milestone Achieved: Define at least 45 primer pairs whose PCR performance in FFPE tissue correlates with the previous determinations in fresh frozen tissue of the same cases and which yields correlating fold-changes between relapse and nonrelapase tumor-adjacent stroma).

This milestone was achieved for 30 primer pairs and not 45 primer pairs. The 30 primer pairs resulted from the application of 10-fold cross validation which is designed to seek the minimum number of genes for forming a classifier.

Major Task 2. Retaining: recalibrating PAM using qPCR values.

Subtask 1: Standardization of performance of FFPE primers by the determination of optimum assay values, minimum detectable value, and precision.

Precision_T
Comparison of replicate wells of a 384 well microfluidics card (192 wells vs. 192 wells)
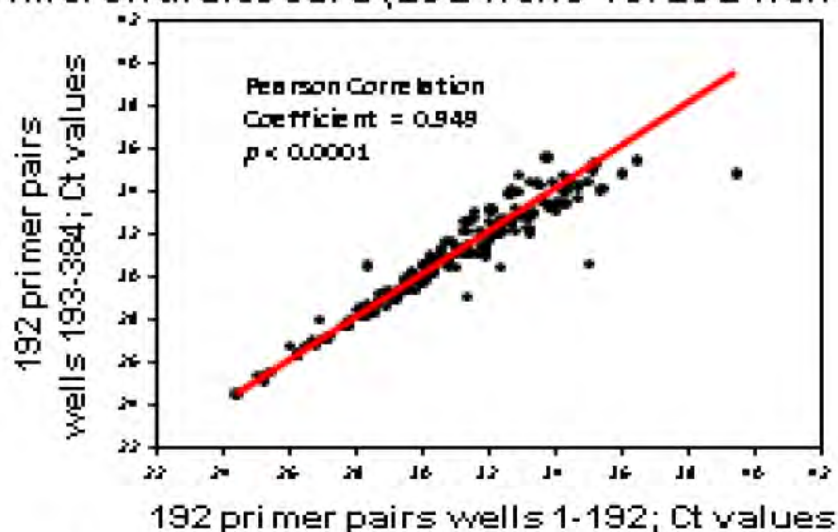
Figure 1. Scatter plot for analysis of precision of technical duplicates.

Progress on Subtask 1. Subtask 1 is completed. Precision for the PCR assay of FFPE RNA by use of our microfluidics 384 well plate was established by examining the precision of measurement of technical replicates (**Figures 1**) and biological duplicates (**Figure 2**). For technical replicates 186 primer pairs for the candidate genes with TagMan reagents plus "housekeeping" primers pairs (192 total prier pairs were applied to the cards twice. These cards are obtained from LifeTechnologies Inc in sets of 20. For the precision analysis RNA made from an FFPE prostate cancer case was applied to two ports of a given card at average of 1.5 ng of RNA per well. The card was then developed in a LifeTechnologies thermocycler and the resulting number of cycles compared for the two duplicated 192 wells (**Figure 1**). The Pearson Correlation Coefficient is 0.95 with a slope of ~ 1.0 indicating very high precision for the performance of the assay.

For the precision of biological duplicates three independent preparation of FFPE RNA was prepared from three separate FFPE blocks of a prostate cancer case and applied to four ports of two cards each containing 192 wells of primer pairs duplicated (**Figure 3**). Thus one card also represented technical replicates for the RNA. The reproducibility for the four housekeeping genes together with the standard deviation is shown. The overall average standard deviation as a percent of mean Ct value is 2.7% or a coefficient of variance of 0.027. Therefore the variability of RNA preparation, loading, thermocycler performance, and card performance is minimal and precision is very high.

Subtask 2: Retaining: recalibrating PAM (program: Prediction Analysis for microarrays) using qPCR values.

Tissue used: Prostate tumor-bearing FFPE blocks from the UCI Pathology archives for relapsed and nonrelapse patients (but not necessarily corresponding to cases of the UCI NCI SPECS recourses).

Figure 2. Comparisons of standard error to the mean for four "housekeeping genes" for triplicate preparations of FFPE RNA of a sample The average coefficient of variance (average of standard deviations/means) is 0.027.
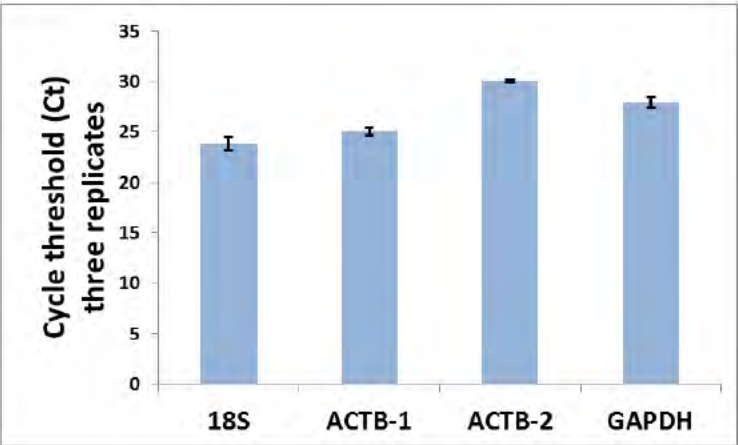

Progress for Subtask 2. Subtask 2 has been completed. We have developed a procedure for migrating the fresh frozen tissue Diagnostic and Prognostic multigene Classifiers to FFPE tissue. **1.** RNA is prepared directly from tumor-adjacent stroma of FFPE tissue. This is accomplished by mounted unstained 20 micron thick recuts of FFPE tissue blocks on plastic histology slides. An adjacent H&E stained section is manually overlaid on the plastic slide and the back of the plastic slide is marked at the location of tumor-adjacent stroma with a fine-point pen. The FFPE tumor-adjacent stroma is then removed from the thick sections on the plastic slides by a 1-2 mm biopsy punch and is used for RNA preparation. **2.** The RNA is used for PCR by applying it to 384 well microfluidic cards preloaded with primer sets and TaqMan reagents as developed in **Subtask 1.** A key strategy was to include primers for genes of the penultimate gene list or those used for 10-fold cross validation during development of the original fresh frozen classifier in order to have replacement genes for those whose FFPE primer pairs that perform poorly during qPCR of FFPE tissue RNA. 186 primer pairs are used for assaying FFPE RNA. **3.** RNA was isolated from tumor-adjacent stroma of 25 prostate cancer cases with known progression-

Figure 3. Kaplan-Meier curve for the classification of patients based on FFPE tumor-adjacent stroma RNA expression according to a 30 gene classifier.

free survival times and used for qPCR on cards with primer pairs for 186 test genes plus 6 housekeeping genes. The results were used for
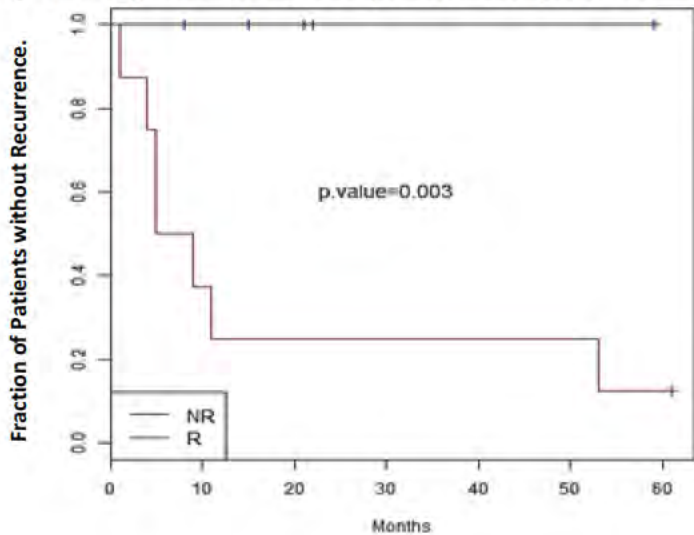
training by 10-fold cross validation process using results for all 186 genes as implemented by the program PAM (Predication Analysis of Microarray data (3). 10-fold cross validation led to a subset of 30 genes of the 186 genes that constitute our preliminary FFPE Prognosis Classifier (**Table 1**). The PCR results for the Prognostic Classifier of recurrence have been used to train a modified FFPE Diagnostic or Prognostic Classifier of recurrence by 10-fold cross validation, as summarized below and in **Figure 3.** The FFPE Prognostic Classifier of recurrence successfully classified the samples with operation characteristic of: 94% accuracy, 100% sensitivity, 87.5% specificity. This is a positive predictive value of 0.9 and a negative predictive value of 1.0.

The corresponding Kaplan-Meier curve is shown in **Figure 3**. The figure shows a highly significant difference between nonrelapse (low risk) patients and relapse (high risk) patients. Moreover the actual classification is nearly perfect - no relapse patients were classified as nonrelapse (**Figure 3**, blue line) and only one nonrelapse patient was classified as relapse (**Figure 3**, red line). These results argue that stroma-based FFPE measurements using primer-pairs adapted from a prognostic classifier for frozen tissue can be used on RNA from FFPE prostate cancer stroma tissue to predict the outcome of radical surgery at the time of diagnosis.

Bioinformatics testing. Bioinformatics testing was used as supportive evidence of the success of **Subtask 2.** We do not have measurements yet on an independent test set of FFPE tissues which would provide a validation. However bioinformatics analyses support the results so far. The FFPE Prognostic Classifier of recurrence was tested on 47 independent fresh frozen samples not used for any of the training steps. These are the tests samples originally used to test the 15-gene fresh frozen patients (21). This test led to operating characteristics with an accuracy = 0.82, sensitivity = 0.64, specificity = 0.95. The test reveals excellent specificity for the detection of patients that subsequently relapse following prostatectomy based on the analysis of stroma RNA values at the time of diagnosis. This argues that the 30-gene FFPE Prognostic Classifier contains genes that function well on RNA derived from tumor-adjacent stroma from FFPE tissue **(Figure 2)** and from fresh frozen tissue.

Milestone Achieved: Definition of the new FFPE Diagnostic Classifier with gene composition and operating characteristics.

This milestone has been achieved for a FFPE Prognostic Classifier which provides a proof-of-principle that multigene classifier for FFPE tissue can be developed with high performance indicating reliable preparation of test RNA and reliable classifier performance.

Specific Aim 3: Aim 3 is to validate the FFPE Diagnostics classifier on an independent test set of clinical cases of known diagnosis in a blinded and randomized trial.

Progress. Not started (for year 2 and 3).

4. **KEY RESEARCH ACCOMPLISHMENTS:**

- Task 1 has been completed. Primers for migrating genes of the diagnostic classifier for application to FFPE RNA have been designed and PCR microfluidics cards obtained. In addition primers of genes used in the development of the prognostic classifier have been designed and PCR microfluidics cards have been obtained. The precision and minimum detectable values of RNA have been defined. The correlation for the measurement of RNA derived from frozen tissues and derived from FFPE tissue for the same set of genes has been defined.

- Task 2 has been completed using the prognostic genes proof-of-principle that multiple genes that accurately classifier prostate cancer cases based on the use of frozen tissue can be migrated to FFPE RNA in order to define multiple genes that accurately classify prostate cancer.

5. CONCLUSION:

The results summarized for **Subtask 2** above have important implications for clinical use. Although the FFPE Prognostic Classifier was developed to provide a proof-of-principle for the methods for migrating genes of classifier for diagnosis and prognosis to FFPE RNA, there are several implications. An accurate multigene FFPE classifier may be applied to patient archived prostatectomy tissue and more generally to patient FFPE diagnostic biopsies. Individual patients would be provided with a classification as being at high or low risk for recurrence following prostatectomy together with the probability of the classification. These results would provide new and crucial patient guidance at the time of diagnosis. For example, patients with high probability for recurrence and who elect surgery may consider immediate adjuvant therapy such as those of refs.(4-7) including an adjuvant therapy protocol developed at UCI (8). Patients with a low probability for recurrence may be advised that the result provides additional reassurance that surgery is potentially curative.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

None to Report.

7. INVENTIONS, PATENTS, AND LICENSES:

None to Report.

8. REPORTABLE OUTCOMES:

Nothing to report.

9. OTHER ACHIEVEMENTS:

Nothing to report.

10. REFERENCES:

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11. **APPENDICES:**

Nothing to report.